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G_1 phase arrest induced by Wilms tumor protein WT1 is abrogated by cyclin/CDK complexes

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ABSTRACT ***WT1*, the Wilms tumor-suppressor gene, maps to the human chromosomal region 11p13 and encodes a transcriptional repressor, WT1, implicated in controlling normal urogenital development. Microinjection of the *WT1* cDNA into quiescent cells or cells in early to mid G_1 phase blocked serum-induced cell cycle progression into S phase. The activity of WT1 varied significantly depending on the presence or absence of an alternatively spliced region located upstream of the zinc finger domain. The inhibitory activity of WT1 was abrogated by the overexpression of cyclin E/CDK2 as well as cyclin D1/CDK4. Furthermore, both CDK4- and CDK2-associated kinase activities were downregulated in cells overexpressing *WT1*, whereas the levels of CDK4, CDK2, and cyclin D1 expression were unchanged. These findings suggest that inhibition of the activity of cyclin/CDK complexes may be involved in mediating the WT1-induced cell cycle block.**

The *WT1* gene was isolated from the human chromosomal region 11p13, a region implicated in the predisposition to the development of Wilms tumor (1, 2). The product of *WT1* is a transcription factor containing a glutamine- and proline-rich region in its amino-terminal domain and four zinc fingers in its carboxyl-terminal domain (3–9). The *WT1* gene generates four major species of WT1 proteins as a combined result of two splicing events, referred to as splices I and II. Splice I inserts 17 aa between the glutamine- and proline-rich amino-terminal domain and the zinc finger domain, whereas splice II inserts 3 aa, Lys-Thr-Ser, between the third and fourth zinc fingers (3, 4). The latter splicing event generates two distinct proteins [WT1-KTS(−) and WT1-KTS(+)] with different DNA-binding specificities (5, 6). WT1-KTS(−) represses the activity of promoters that contain a specific consensus sequence, including the promoters of insulin-like growth factor II and platelet-derived growth factor A chain (7–9).

Expression of *WT1* is restricted to a limited set of tissues including fetal kidney, testis, and ovary (1, 2, 10) and is believed to play an important role in the development of these tissues. Indeed, analysis of mice carrying a targeted mutation in *WT1* has shown that this gene plays a crucial role in normal early urogenital development (11). On the other hand, *WT1* was found to be highly expressed in human hematopoietic malignancies (12–14). Of particular interest is the correlation between the levels of *WT1* gene expression and a poor prognosis in acute leukemia (14). Moreover, WT1 was found to be a marker for the detection of minimal residual disease in acute leukemia.

In about 10% of Wilms tumors, point mutations and small deletions in the zinc finger region of WT1 have been detected (15, 16), confirming that the loss of WT1 function is important for the development of Wilms tumor. Moreover, WT1 can inhibit colony formation in a cell line derived from Wilms tumor (17). In the present study, we examined the action of WT1 on cell cycle progression and found that overexpression of *WT1* at early to mid G_1 phase blocked cell cycle progression into S phase. Further, we found that CDK-4 and CDK-2 kinase activities were downregulated in cells overexpressing *WT1* and that overexpression of cyclin/CDK abrogates the effect of WT1. These results suggest a link between WT1 function and the regulation of cell cycle-dependent kinases.

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MATERIALS AND METHODS

Construction of *WT1* Expression Plasmids. The *WT1* expression plasmids pDOLWT1-17(+)-KTS(+), -17(−)-KTS(+), -17(+)-KTS(−), and -17(−)-KTS(−) were generated by inserting the full-length protein-coding region (*Eco*47III–*Hin*cII fragments) of the four variant WT1 cDNAs into the *Mlu* I site of the pDOL vector, which carries the murine leukemia virus long terminal repeat and a neomycin-resistance gene, by use of an *Mlu* I linker. WT1-17(+)-KTS(+) encodes WT1 with splices I and II; WT1-17(−)-KTS(+), WT1 without splice I but with splice II; WT1-17(+)-KTS(−), WT1 with splice I but without splice II; and WT1-17(−)-KTS(−), WT1 without splices I and II. The *WT1* expression plasmids pMEWT1-17(+)-KTS(+), -17(−)-KTS(+), -17(+)-KTS(−), and -17(−)-KTS(−) were generated by inserting the full-length protein-coding region (*Eco*47III–*Hin*cII fragments) of the four variant WT1 cDNAs between the *Eco*RI and *Not* I sites of the pME18S vector, which carries the SRα promoter. The mutant WT1-17(+)-KTS(+), encoding Gly instead of Asp-396, was generated by site-directed mutagenesis (18) with the synthetic oligonucleotide 5′-TTCTCCCGGTCCGGCCACCTGAAGACCCACACC-3′.

Antibodies. Anti-WT1 antibodies were prepared by immunizing rabbits with a synthetic peptide (aa 177–192 of WT1) and purified by affinity chromatography on a column to which the synthetic peptide had been covalently linked. Anti-β-galactosidase polyclonal antibodies, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and rhodomine isothiocyanate-conjugated goat anti-mouse IgG were obtained from Cappel. Anti-bromodeoxyuridine (BrdUrd) monoclonal antibody (mAb) BU-4 was from Takara (Tokyo). Anti-CDK2 antibodies and anti-cyclin D1 antibodies were prepared by immunizing rabbits with synthetic peptides corresponding to the carboxyl-terminal 12 aa of human CDK2 or cyclin D1. Anti-CDK4 antibodies were obtained from Santa Cruz Biotechnology. Biotin-conjugated monoclonal anti-decay accelerating factor (DAF) mAb B-1A10 was from T. Kinoshita (Osaka University). Phycoerythrin-conjugated streptavidin was obtained from Biomedia (Foster City, CA). Alkaline phosphatase-conjugated anti-rabbit antibodies were purchased from Promega.

Colony Formation Assay. Monkey CV-1 cells (2×10^5 per 6-cm dish) were transfected with 2 μg of the *WT1* expression

Abbreviations: DAF, decay accelerating factor; mAb, monoclonal antibody.

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plasmids (pDOLWT1 series) by using Lipofectin (BRL). Twenty-four hours after transfection, cells were trypsinized, diluted 1:10, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and geneticin (500 μg/ml) for 3 weeks. The plates were fixed and stained with Giemsa reagent.

Microinjection. Mouse NIH 3T3 cells, grown on coverslips (10^4 cells per cm^2), were cultured in DMEM containing 0.4% calf serum for 24 hr and then microinjected with the normal or mutated *WT1* expression plasmid (pMEWT1 series) or β-galactosidase expression plasmid (pBAβ-galactosidase) (200 μg of DNA per ml). After incubation for 24 hr in the same conditions, medium was replaced with fresh DMEM containing 10% calf serum and 50 μM BrdUrd, and the cells were incubated for another 18 hr. Cells were fixed in 3.7% formaldehyde in phosphate-buffered saline for 30 min, dehydrated with 100% methanol for 10 min, and then treated with 2 M HCl for 10 min. WT1 protein was detected with anti-WT1 antibodies and fluorescein-conjugated goat anti-rabbit IgG. BrdUrd was detected with mAb BU-4 followed by rhodamine-conjugated goat anti-mouse IgG. For the time-course experiment, cells were injected with DNA at the indicated times (see Fig. 1 *Lower*) and labeled with BrdUrd until 18 hr after serum stimulation.

Flow Cytometry. NIH 3T3 cells (3×10^6) were cotransfected with pMEDAF (20 μg/0.8 ml), a vector containing the human DAF cDNA, and WT1-17(+)-KTS(−) (40 μg/0.8 ml) by electroporation using a Gene Pulser (Bio-Rad) (960 μF, 260 mA) (19, 20). Cells were cultured in DMEM with 10% calf serum for 5 hr and then in DMEM with 0.4% calf serum for 48 hr. Subsequently, the medium was replaced with fresh DMEM containing 10% calf serum, and the cells were incubated for another 12 hr. Cells were then stained with biotin-conjugated anti-DAF mAb B-1A10 and phycoerythrin-conjugated streptavidin, and immunostained cells were separated by flow cytometry on a FACS Vintage Machine (Becton Dickinson).

***In Vitro* Kinase Assay and Western Blot Analysis.** DAF^+ NIH 3T3 cells separated by flow cytometry were lysed in 50 mM Tris, pH 7.2/1% (vol/vol) Nonidet P-40/0.15 M NaCl/5 mM dithiothreitol/0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride with leupeptin at 5 mg/ml and subjected to immunoprecipitation with anti-CDK2 antibodies as described (21). The immunocomplexes were incubated with 1 μg of bacterially produced glutathione *S*-transferase–retinoblastoma (RB) protein fusion protein in 50 μl of 50 mM Tris, pH 7.2/10 mM $MgCl_2$/1 mM dithiothreitol/20 μM [γ-^{32}P]ATP (185 kBq) for 10 min at 25°C for assay of CDK4-associated RB kinase activity (21). Samples were analyzed by SDS/12% PAGE followed by autoradiography.

For Western blot analysis, lysates obtained as described above were resolved by SDS/12% PAGE and transferred onto poly(vinylidene difluoride) membranes (Immobilon-P; Millipore). Blots were probed with antibodies to CDK4, CDK2, cyclin D1, or WT1 and subsequently with alkaline phosphatase-conjugated second antibodies.

RESULTS

We constructed plasmids expressing each of the four possible spliced *WT1* gene products arising from splices I and II. When each of the four *WT1* expression constructs was transiently transfected into monkey CV-1 cells, we could detect the respective WT1 proteins synthesized from the constructs by Western blotting analysis (data not shown). CV-1 cells transfected with a control expression vector, lacking the WT1 cDNA, expressed no detectable WT1.

To assess the ability of WT1 to inhibit cell proliferation, we performed a colony formation assay. The four *WT1* constructs were transfected into CV-1 cells, and Geneticin-resistant colonies were counted 3 weeks later. Cells transfected with the *WT1* expression plasmid formed 2–10 times fewer colonies than those transfected with the control expression vector (Table 1). The presence of the splice I insert appeared to have a more dramatic effect in inhibiting colony formation regardless of the presence or absence of the splice II insert. Western blot analysis showed no expression of the exogenous WT1 protein in any of 10 clonal lines established from CV-1 cells transfected with the *WT1* cDNA with the splice I insert, whereas low expression of the WT1 protein was detected in 20% of clones established from cells transfected with the *WT1* cDNA lacking the splice I insert. These results suggest that WT1 inhibited the growth of CV-1 cells.

We next tested whether WT1 blocks cell cycle progression at a specific point. For this purpose, we examined the effect of *WT1* expression on serum-induced cell cycle progression into S phase. NIH 3T3 cells were brought to quiescence by culturing in medium containing 0.4% serum for 24 hr. Cells were then microinjected with the *WT1* expression plasmid and cultured for a further 24 hr in 0.4% serum. Fresh medium supplemented with 10% serum was then added to induce synchronous cell cycle progression, and BrdUrd was added to measure DNA synthesis. After an additional incubation for 18 hr, microinjected cells were identified by staining for the WT1 protein, and cells that entered S phase were identified by staining for BrdUrd incorporation. While almost all cells microinjected with a vector containing the β-galactosidase cDNA incorporated BrdUrd, cells microinjected with the *WT1* cDNA showed significantly lower BrdUrd incorporation (Fig. 1 *Upper*; Table 2). Consistent with the results of the colony formation assay, microinjection of *WT1* variants containing the splice I insert had a more pronounced inhibitory effect than microinjection of those lacking this insert. In contrast, a mutant *WT1* cDNA encoding Gly instead of Asp-396, a mutation identified in one case of sporadic Wilms tumor (15), did not show any inhibitory activity. We also examined the effect of WT1 on NIH 3T3 cells arrested in early S phase by treatment with aphidicolin. The WT1 cDNA was unable to inhibit BrdUrd incorporation (Fig. 1 *Lower*), suggesting that WT1 does not directly block DNA synthesis.

The inhibitory effect of WT1 was also observed with various cell lines: asynchronously growing mouse NIH 3T3 cells and monkey CV-1 and COS-7 cells, which express endogenous WT1 at undetectable or very low levels, as well as mouse F9 and P19 embryonal carcinoma cells, which do express endogenous WT1 (Table 2).

The results of the above experiments suggested that WT1 blocks cell cycle progression through G_1 phase. Thus we next tried to define more precisely the time point at which WT1 exerts its inhibitory activity. At various times after the addition of serum, NIH 3T3 cells were injected with the *WT1* cDNA containing both splices I and II [WT1-17(+)-KTS(+)] and DNA synthesis was scored by measuring BrdUrd incorporation. WT1 synthesized from the injected cDNA was detectable

Table 1. Colony formation after transfection with *WT1* expression plasmid

Transfected DNA	No. of Geneticin-resistant colonies (%)	
	Experiment 1	Experiment 2
pDOL	260 (100)	308 (100)
pDOLWT1-17(+)-KTS(+)	50 (19.2)	38 (12.3)
pDOLWT1-17(−)-KTS(+)	114 (43.8)	115 (37.7)
pDOLWT1-17(+)-KTS(−)	25 (9.6)	31 (10.1)
pDOLWT1-17(−)-KTS(−)	101 (38.8)	54 (17.5)

CV-1 cells were transfected with the indicated plasmids and cultured in the presence of Geneticin (500 μg/ml) for 3 weeks. Data obtained from two independent experiments are shown.

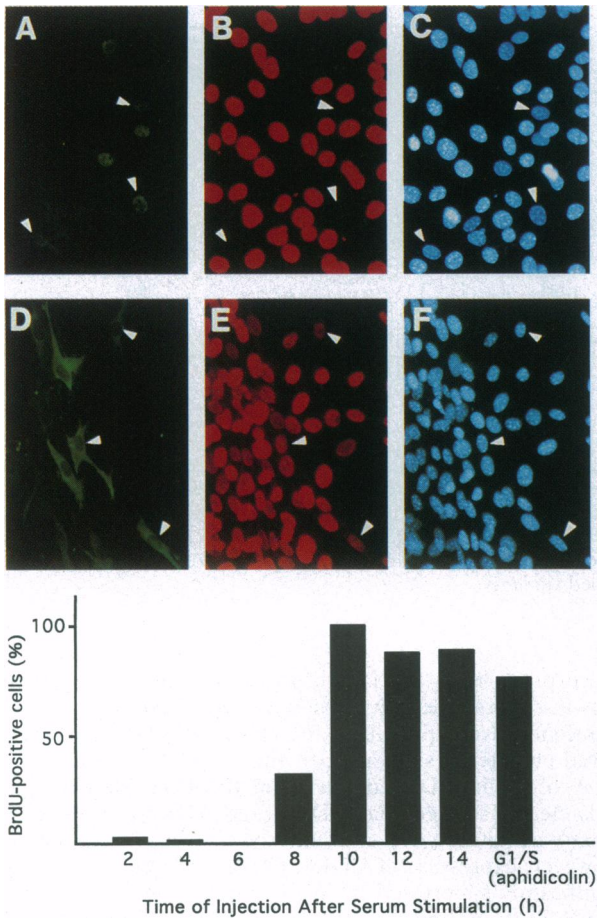

FIG. 1. Effect of WT1 on cell cycle progression. (*Upper*) Microinjection of *WT1* cDNA inhibits serum-induced G_0/G_1-to-S transition. Serum-deprived NIH 3T3 cells microinjected with *WT1* (*A–C*) or β-galactosidase (*D–F*) expression plasmid were restimulated with serum and then visualized for WT1 (*A*) or β-galactosidase (*D*) or BrdUrd incorporation (*B* and *E*) or stained with 4′,6-diamidino-2-phenylindole (*C* and *F*). White arrowheads point to three representative cells. Only the results obtained with the WT1-17(+)-KTS(+) cDNA are shown. (*Lower*) Microinjection of the WT1 cDNA at various time points through G_1 to S phase. Serum-starved NIH 3T3 cells were microinjected with the WT1-17(+)-KTS(+) cDNA at the indicated times after stimulation with 10% calf serum. Histogram shows the percentage of BrdUrd-positive cells (70 cells were counted). Data obtained with cells arrested at the G_1/S boundary are also shown.

in the nucleus at least 2 hr after microinjection. A time course with uninjected cells showed that the number of BrdUrd-positive cells began to increase about 13 hr after serum addition and reached the maximum by 14 hr (data not shown). Injection of the *WT1* cDNA at 8 hr after serum addition could still inhibit BrdUrd incorporation, but injection at 10 hr was ineffective (Fig. 1 *Lower*). Similar results were obtained with the *WT1* cDNA containing splice I but not splice II (data not shown). These results suggest that WT1 acts in mid to late G_1 phase.

To elucidate the mechanism by which WT1 blocks the cell cycle, we examined whether overexpression of molecules important for cell cycle regulation could overcome the inhibitory effect of WT1 [WT1-17(+)-KTS(+) and WT1-17(+)-KTS(−)]. We first performed comicroinjection experiments with the adenoviral E1A, simian virus 40 large tumor antigen, and papilloma E6 and E7 genes but found no effect on WT1 inhibitory activity, suggesting that inhibition of RB or p53 is not important for the action of WT1. Similarly, comicroinjection of c-*fos*, CDK2, CDK4, or cyclin D1 cDNA had no effect on the inhibitory activity of WT1. However, comicroinjection of CDK4 cDNA along with cyclin D1 cDNA, or CDK2 cDNA along with cyclin E cDNA, abrogated WT1 inhibition [Table 3; only the data obtained with WT1-17(+)-KTS(+) are shown]. Microinjection of the cyclin E cDNA alone also had a partial effect in abrogating WT1 inhibition.

The results of the comicroinjection experiments suggested that cyclin/CDK complexes may be functionally involved in the WT1-mediated cell cycle block. We therefore examined whether *WT1* expression alters the levels of cyclin/CDK and/or activity. Since we were unable to establish cell lines in which WT1 was inducibly expressed, we performed experiments using transiently expressed WT1. To separate cells that expressed the transfected WT1 plasmid (about 5% of total) from those that did not, we cotransfected a cDNA encoding the surface-expressed DAF, enabling us to separate cells by flow cytometry based on DAF immunofluorescence. Serum-deprived NIH 3T3 cells which had been cotransfected with the DAF cDNA and a cDNA encoding a version of WT1, WT1-17(+)-KTS(−), were induced to enter the cell cycle by the addition of 10% serum. The cells were separated by flow cytometry 12 hr later and subjected to Western blot analysis and kinase assay (Fig. 2*A*). While almost all of the untransfected cells or cells transfected with DAF alone took up BrdUrd, <5% of the cells expressing the transfected *WT1* were able to incorporate BrdUrd. Ectopic WT1 was readily detectable in the DAF^+ cells cotransfected with the *WT1* cDNA (Fig. 2*B*). CDK4 and CDK2 were immunoprecipitated and assayed for their abilities to phosphorylate bacterially produced RB (Fig. 2). DAF^+ cells cotransfected with *WT1* cDNA displayed very low levels of CDK4- and CDK2-associated kinase activities, in contrast to both the untransfected cells and cells transfected with DAF cDNA alone, whose kinase activities were enhanced by the addition of serum. However, Western blot analysis revealed that the levels of CDK4, CDK2, and cyclin D1 were not significantly affected by WT1 (Fig. 2*B*). The faster migrating, active form of CDK2 was detected in the *WT1*-expressing cells as well as in the control cells, though its amount was partially reduced. The level of cyclin E was difficult to assess, since the anti-cyclin E antibodies available to us failed to detect a definitive band of cyclin E.

DISCUSSION

Using colony formation assays and microinjection experiments, we demonstrated that WT1 inhibits cell growth by blocking cell cycle progression from G_1 to S phase. The effect of WT1 was seen with cells which do or do not contain endogenous WT1. By contrast, the mutant WT1 which was identified in one case of sporadic Wilms tumor did not show cell cycle-blocking activity. These results are consistent with the observation that inactivation of WT1 results in the development of Wilms tumor (1, 2, 15–17). Physiologically, *WT1* is expressed in the fetal kidney, urogenital precursors, and many supportive structures of mesodermal origin (10, 12). Thus, WT1 may play an important role in the regulation of cell cycle progression in these tissues.

Since WT1 is a zinc finger-containing transcriptional repressor (7–9), suppression of cellular genes that are essential for cell cycle progression could be responsible for the observed WT1-mediated inhibition of cell growth. Consistent with this notion, variants of WT1 containing the splice I insert have stronger transcriptional repressor activity as well as cell cycle blocking activity than those without this insert (unpublished data); there is a close relationship between the cell cycle-blocking activity and the transcriptional repressor activity. Importantly, microinjection of the *WT1* cDNA at 8 hr after serum addition could still inhibit cell cycle transition to the S phase. This finding suggests that repression of genes expressed

Table 2. Effect of *WT1* expression on BrdUrd incorporation

Condition	Cell line	Injected DNA	No. of WT1$^+$ cells	No. of BrdUrd$^+$ cells	% BrdUrd$^+$	% BrdUrd$^+$ in uninjected cells*
Synchronized growth	NIH 3T3	pMEWT1-17(+)-KTS(+)	127	4	3.1	91
		pMEWT1-17(−)-KTS(+)	103	58	56.3	92
		pMEWT1-17(+)-KTS(−)	282	10	3.5	89
		pMEWT1-17(−)-KTS(−)	115	59	51.3	88
		pMEmutant WT1-17(+)-KTS(+)	51	38	74.5	87
		pBAβ-galactosidase	149†	123	82.5	89
Asynchronous growth	NIH 3T3	pMEWT1-17(+)-KTS(+)	40	0	0.0	78
	CV-1	pMEWT1-17(+)-KTS(+)	75	0	0.0	96
	COS-7	pMEWT1-17(+)-KTS(+)	37	2	5.4	76
	F9	pMEWT1-17(+)-KTS(+)	22	0	0.0	100
	P19	pMEWT1-17(+)-KTS(+)	18	0	0.0	100

Serum-starved NIH 3T3 cells were microinjected with the indicated plasmid, restimulated with 10% calf serum, and then labeled with BrdUrd and were stained for WT1 and BrdUrd; nuclei were visualized with 4′,6-diamidino-2-phenylindole. Asynchronously growing NIH 3T3 cells, monkey kidney fibroblast cell lines CV-1 and COS-7 (CV-1 expressing simian virus 40 large tumor antigen), and mouse embryonal carcinoma cell lines F9 and P19 were also microinjected with the *WT1* expression plasmid, labeled with BrdUrd for 24 hr, and then examined similarly.

*One hundred uninjected cells were scored.

†Cells were stained with an anti-β-galactosidase antibody.

prior to this time point by WT1 is not essential for cell cycle arrest.

Our comicroinjection experiments showed that overexpression of cyclin E plus CDK2 or cyclin D1 plus CDK4 abrogated WT1-mediated G_1 growth arrest. The D-type cyclins, which primarily associate with and activate CDK4, appear in early to mid G_1 phase, whereas cyclin E, which activates CDK2, is synthesized near the G_1-to-S transition. Furthermore, cyclin D/CDK4 and cyclin E/CDK2 are believed to function as the master regulators of cell cycle progression through G_1 phase (22, 23). Hence, our findings suggest that cyclin/CDK complexes may play an important role in the pathway involving WT1. For example, WT1 may function upstream of cyclin/CDK and may block progression of cells through G_1 by inhibiting the activities of cyclin/CDK complexes. However, it is also possible that cyclin/CDK functions upstream of WT1 and downregulates the inhibitory activity of WT1, although WT1 itself has no site for phosphorylation by cyclin/CDK.

To discriminate between the possibilities described above, we sought to examine CDK4- and CDK2-associated kinase activities in the *WT1*-overexpressing cells. Since it is impossible to perform biochemical characterization with *WT1*-microinjected cells, we tried to establish cell lines that inducibly overexpress *WT1*. We failed, presumably due to the strong growth-suppressive activity of WT1. We therefore used cells transiently overexpressing *WT1* and DAF cDNA, which allowed cytometric separation of the transfected cells. Kinase assay of cyclin/CDK complexes in the *WT1*-overexpressing cells clearly showed that CDK4- and CDK2-associated RB kinase activities were not induced by serum stimulation. However, expression of CDK4, CDK2, or cyclin D1 was not significantly suppressed by WT1, eliminating the possibility that WT1 simply arrested cells before they become competent to produce these molecules. Thus, WT1 may block cell cycle progression by inhibiting the activities of cyclin/CDK complexes.

CDK activation requires the formation of a complex with cyclin and phosphorylation of a conserved threonine residue by CDK-activating kinase, a kinase composed of CDK7 and cyclin H (24–26). Thus, WT1 may act by interfering with either cyclin/CDK complex assembly or the process of activation. Western blot analysis of CDK2 in the *WT1*-expressing cells showed that the amount of the faster migrating, active form was decreased partially (Fig. 2), suggesting that the decrease in CDK2 activity may be partly ascribed to a decrease in the amount of the active form. Another mechanism for regulation

Table 3. Effect of overexpression of cell cycle-regulating genes on WT1-mediated cell cycle arrest

DNA coinjected with pMEWT1-17(+)-KTS(+)	No. of WT1$^+$ cells	No. of BrdUrd$^+$ cells	% BrdUrd$^+$ in injected cells	% BrdUrd$^+$ in uninjected cells*
None	127	4	3.1	91
pMEc-fos	45	2	4.4	93
pMEcyclin D1	53	1	1.9	99
pMEcyclin E	81	17	21.0	87
pMECDK2	56	0	0.0	93
pMECDK4	35	0	0.0	95
pMECDK2 and pMEcyclin E	45	45	100.0	98
pMECDK4 and pMEcyclin D1	25	21	84.0	88
Simian virus 40 large tumor antigen	58	4	6.9	89
Adenoviral E1A	26	2	7.7	91
Papilloma E6	42	0	0.0	92
Papilloma E6 and E7	32	0	0.0	90

NIH 3T3 cells were synchronized, microinjected with the indicated plasmids (200 μg/ml) and stained for WT1 and BrdUrd. Expression of coinjected genes was confirmed by staining the cells with the corresponding specific antibodies in the parallel experiments.

*One hundred uninjected cells were scored.

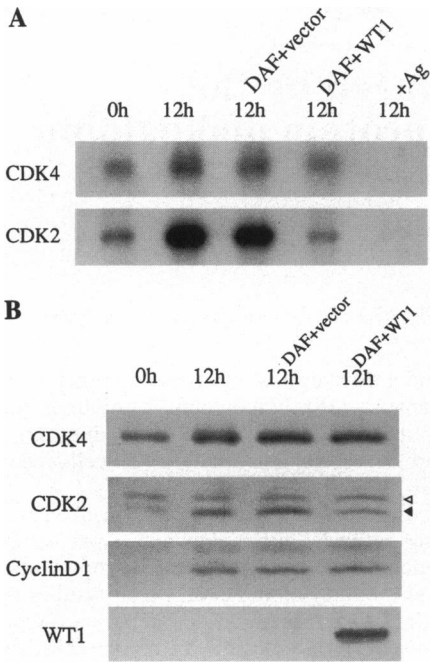

FIG. 2. (*A*) Effect of WT1 on the activity of cyclin/CDK. NIH 3T3 cells were transfected with the control expression vector or the WT1-17(+)-KTS(−) expression construct along with the DAF expression construct. Cells were serum-starved for 48 hr and then induced to enter the cell cycle by the addition of 10% serum. After 12 hr, the transfected cells were separated by flow cytometry based on DAF immunofluorescence. CDK4 and CDK2 were immunoprecipitated from the separated cells (10^5 cells) and were subjected to RB kinase assay. Kinase assays were also performed with control (untransfected) NIH 3T3 cells harvested at 0 and 12 hr after serum addition. When the antibodies that had been preabsorbed with antigens (+Ag) were used, kinase activity was not detected in the precipitates prepared from control NIH 3T3 cells cultured in the presence of 10% serum for 12 hr. (*B*) Effect of WT1 on the levels of CDK4, CDK2, and cyclin D1. Cells used for the kinase assay in *A* were also subjected to Western blot analysis using anti-CDK4, -CDK2, -cyclin D1, or -WT1 antibodies. White and black arrowheads indicate inactive and active CDK2, respectively.

of cyclin/CDK activity is the formation of complexes with the class of small CDK inhibitor proteins such as p21 (also known as SDI1, WAF1, CIP1, CAP20, or PIC1), $p16^{INK4}$, $p15^{INK4B}$, and $p27^{Kip1}$ (22, 23). In this regard, p53 has been shown to block the cell cycle by inducing the expression of p21 (27–30, 34). Also, transforming growth factor β-induced cell cycle arrest has been suggested to be mediated by p15 and p27 (31–33). Thus, we speculate that WT1 may also regulate the cell cycle by altering the expression of genes involved in controlling the activity of cell cycle-dependent kinase complexes.

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